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SEX DIFFERENCES IN SULFATION AND GLUCURONIDATION OF PHENOL, 4-NITROPHENOL AND *N*-HYDROXY-2-ACETYLAMINOFLUORENE IN THE RAT *IN VIVO*

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Abstract—Sulfation and glucuronidation of phenol, 4-nitrophenol (4NP) and *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF) were studied in adult (60 days) male and female rats.

Within 3 hours almost 50% of a dose of phenol was excreted in urine as phenyl sulfate; male rats excreted slightly more phenyl sulfate than females. This probably was due to a slower excretion of phenyl sulfate by the females. No sex difference in glucuronidation of phenol was found.

Over a period of 24 hours male and female rats excreted almost 35% of a dose of 4NP as 4NP-sulfate in urine and almost 40% as 4NP-glucuronide. No differences in the excretion of 4NP-conjugates were found between sexes.

However, almost twice as much of a dose of N-OH-AAF was excreted after 4 hours as the N-O-glucuronide in bile and urine in female than in males. On the other hand, females excreted less of the AAF-glutathione conjugates that are derived from the reaction of AAF-N-sulfate with glutathione *in vivo* [Meerman *et al.*, *Chem.-Biol. Interactions*, **39**, 149, 1982] in bile, than males. This indicates that sulfation of N-OH-AAF is less active in females than in males. Most likely, sulfation of the phenols is catalyzed by a different sulfotransferase than that of N-OH-AAF.

Sulfation is a major route of biotransformation by which many xenobiotics are converted to conjugates that are readily excreted in bile and urine. In most cases, stable sulfate esters are formed and potentially toxic compounds are detoxified by this conjugation process. However, some compounds are converted into unstable sulfate conjugates which are thought to be responsible for the toxic and/or carcinogenic properties of the parent compound [1].

It has been suggested that male rats are more susceptible to the hepatotoxic and carcinogenic effects of *N*-hydroxy-2-acetylaminofluorene (N-OH-AAF) [2, 3], *N*-hydroxy-*N*-methylaminoazobenzene [4] and 1′-hydroxy-safrole [5] than females due to a higher hepatic sulfotransferase activity which converts them to reactive, electrophilic species capable of reacting with DNA. Sex differences in sulfation may also exist for other compounds [5] but *in vivo* data are scarce. Sex hormones are most likely involved in the development of sulfotransferase activity towards N-OH-AAF: in weanling rats, sulfation of N-OH-AAF is low in both males and females [6] while testosterone was able to increase sulfotransferase activity in the liver cytosol of males as well as females. It was decreased in both sexes by 17β-estradiol.

Because very limited *in vivo* data exists on sex differences in sulfation of xenobiotics, we studied the sulfation of phenol and 4-nitrophenol, and for comparison of N-OH-AAF, in rats of both sexes.

MATERIALS AND METHODS

Animals. SPF Wistar rats of the strain of the Department of Pharmacology, University of Leiden, were used at approximately 60 days of age. They had free access to food and water. The rats were anesthetized with pentobarbital sodium (60 mg/kg i.p.) and the bile duct, external jugular vein and urine bladder were catheterized. An infusion of mannitol (75 mg/ml in water) was given via the jugular vein at a rate of 3.8 ml/hr to enhance urine production. Body temperature was kept between 37.5° and 38.5°. Phenol (133 μmol/kg) was injected into the catheterized jugular vein in a volume of 1 ml of saline/kg body weight; urine and bile were collected for 3hr in two fractions of 15 min during the first $\frac{1}{2}$ hr and fractions of 30 min for the remaining period.

In the experiments with N-OH-AAF, rats were treated similarly except for the catheterization of the jugular vein. Instead, N-OH-AAF (60 μmol/kg) was injected in a lateral tail vein. The injection solution was prepared as described before [7]. Bile and urine were collected on ice for 4 hr.

4NP (60 μmol/kg) was injected in a lateral tail vein in a volume of 1 ml of saline/kg of bodyweight. The rats were placed in metabolism cages and urine was collected on ice for 24 hr in two 6 hr and one 12 hr fractions.

Materials. 4-Nitrophenol, >99% pure, was from Fluka (Buchs, Switzerland). 4-Nitrophenyl-β-D-glucuronide was from Boehringer (Mannheim, W. Germany). Potassium 4-nitrophenyl sulfate and TLC plates (Silicagel 60 F_{254} Alufoil plates) were from Merck (Darmstadt, W. Germany). [^{14}C]-phenol (5.6 mCi/mmol; dissolved in benzene) was from New England Nuclear (Dreieich, W. Germany); an equal part of water was added and the two layers were

mixed intensively. The benzene layer was removed with a stream of nitrogen and the aqueous layer was mixed with a solution of unlabeled phenol in saline to obtain the desired specific radioactivity of 7.5 μCi/mmol.

[ring-^{3}H]-N-OH-AAF (spec. act. 83 mCi/mmol; synthesized as described before [8]) was mixed with unlabeled N-OH-AAF to obtain the desired specific radioactivity of 1 mCi/mmol.

Hydrocount, methanol (HPLC-reagent grade) and all other reagents were from J. T. Baker Chemical Comp. (Deventer, The Netherlands).

The liquid scintillation cocktail Flo-Scint II was from Radiomatic Instruments & Chem. Co. (Tampa, FL), and Plasmasol was from Packard Instrument Co. (Downers Grove, IL).

Determination of phenyl sulfate and glucuronide in bile and urine. Phenyl conjugates were determined by TLC as described before [9]. The TLC plates were cut into small pieces (1 × 1 cm) and transferred into scintillation vials. Radioactivity was determined by liquid scintillation counting after the addition of 6.0 ml of Hydrocount. Recovery of radioactivity applied on the TLC plates was 97.7 ± 2.2% (mean ± SEM; N = 14).

Determination of 4NP-glucuronide and 4NP-sulfate in bile and urine. Bile or urine samples (5 μl) were separated on a 0.3 × 7.5 cm 7-Nucleosil-phenyl column (Machery and Nagel, Düren, W. Germany) connected with a 0.39 × 15 cm 5Sperisorb-ODS II column (Phase Separations, Queensferry, Clwyd, U.K.) that was eluted with acetonitrile:aqueous 10 mM potassium phosphate buffer pH 3.00 (7:93) at a flow of 1 ml/min. The eluent was delivered by a Waters M45 pump (Waters Ass., Milford, MA). The effluent was monitored at 254 nm with a Waters 440 absorbance detector. Identification of peaks was by comparison of retention times with those of original compounds. 4NP-glucuronide eluted at 9.5 min and 4NP-sulfate at 10.75 min. A linear correlation ($r > 0.9995$) between the amount injected and peak-height was obtained in the range of 1–10 nmol. Samples which contained more than 1.5 mM of the conjugates were diluted with water before injection.

Determination of AAF-N,O-glucuronide, 1-GS-AAF and 3-GS-AAF. N-OH-AAF metabolites in bile and urine were determined as described before [8] except for the dimensions of the column which were 0.3 × 15 cm and the flow which was 0.6 ml/min. Radioactivity in the effluent was measured after the admixture of 1.8 ml of Flo-Scint II/min with a Flow-One model 1C detector (Radiomatic Instruments & Chem. Co., Tampa, FL) equipped with a 400 μl cell. Quench correction was made by injection of known amounts of ^{3}H.

Determination of radioactivity covalently bound to macromolecules in the rat liver. Livers were homogenized and macromolecules were purified as described before [7]. Of the dried macromolecular product, 10–15 mg was dissolved in 0.5 ml of a 1 M aqueous NaOH solution and the concentration of protein was determined by the method of Lowry *et al.* [10]. A 200 μl aliquot was taken and the radioactivity was determined after the addition of 6 ml of Plasmasol.

RESULTS

Phenol

Phenol was excreted mostly in urine, mainly in the form of the conjugates. Only a minor percentage of the dose, mainly in the form of the glucuronide conjugate, was excreted in bile. The ratio between the sulfate and the glucuronide conjugate was the same in both sexes, indicating that there were no major sex differences in conjugation (Table 1).

Preliminary data suggest that the sex difference in the recovery of phenol was due to a slower elimination of the conjugates in the urine because urinary elimination half-lives were some 30% longer in females than in males.

4-Nitrophenol

Adult male rats excreted 33% of a dose of 4NP as 4NP sulfate in urine (Table 2); females excreted the same amount (34%). The amount of 4NP-glucuronide that was excreted by both sexes was also the same (males 39%; females 37%).

N-OH-AAF

Adult male rats excreted almost 26% of a dose of N-OH-AAF as the N,O-glucuronide conjugate (Table 3). Females, however, excreted a much larger part of the dose as the glucuronide conjugate (43%). In contrast, females excreted much less of the 1-GS and 3-GS-glutathione conjugates in bile as compared to males (Table 3). The covalent binding of N-OH-AAF to rat liver macromolecules also showed a great sex difference: in males 870 ± 10 pmol of N-OH-AAF became bound per mg of protein (N = 4) whereas in females this was 380 ± 80 pmol/mg (N = 6; significantly different by Fisher's exact test at $P < 0.01$).

DISCUSSION

Sulfation of the carcinogen N-OH-AAF converts this compound into a very reactive metabolite that is capable of reacting with DNA, RNA and protein in the liver of rodents (which is one of the target organs for its carcinogenic action). Sex differences in sulfation of N-OH-AAF exist and this might explain the higher susceptibility of males for carcinogenesis by N-OH-AAF and related compounds as compared to females [1].

It has been reported that a sulfotransferase isolated from rat liver which accepts N-OH-AAF as a substrate, also accepts 4NP [13]. Therefore, we have investigated whether sex differences in sulfation in rats *in vivo* also exist for phenol and 4NP.

The results presented in this study show that there are no sex differences in sulfation of phenol and 4NP in the rat *in vivo*. Preliminary results indicate that also for harmol (7-hydroxy-1-methyl-9*H*-pyrido[3,4-*b*]indole), there are no sex differences in sulfation.

We have also determined the sulfation of N-OH-AAF in our strain of rats in order to verify whether a sex difference in sulfation of this carcinogen exists in our strain of rats as has been reported for other strains. Unfortunately, sulfation of the carcinogen N-OH-AAF cannot be measured *in vivo* by measuring the formation of the *N*-sulfate conjugate of

Table 1. The excretion of phenyl sulfate and phenyl glucuronide in bile and urine of male and female rats after the i.v. administration of [^{14}C]-phenol

	Bodyweight (g)		Total radio-activity	Phenyl sulfate	Phenyl glucuronide	S/G ratio
			% of the dose			
Males	360 ± 0	Urine	91.2 ± 4.5	53.0 ± 2.1	44.6 ± 2.9	1.15
		Bile	3.6 ± 1.2	n.d.	1.6 ± 0.8	
Females	235 ± 1	Urine	87.3 ± 2.6*	44.4 ± 3.0*	39.5 ± 3.9	1.04
		Bile	4.1 ± 0.3	n.d.	3.3 ± 0.5	

Rats received an i.v. dose of [^{14}C]-phenol (133 μmol/kg; spec. act. 7.5 μCi/mmol). Bile and urine were collected during 3 hr. n.d.: Means not detectable (less than approx. 0.7% of the dose). The total excretion of conjugates was calculated from the amounts excreted in the separate fractions. Results are expressed as mean ± SEM of 3 rats. * Indicates different from male rats at $P < 0.06$ by Fisher's randomization test [15].

Table 2. The excretion of 4NP sulfate and 4NP glucuronide in urine of male and female rats

	Body weight (g)	4NP sulfate	4NP glucuronide	Total
		% of the dose		
Males	349 ± 9	33.2 ± 4.6	39.4 ± 5.6	72.5 ± 10.1
Females	245 ± 4	34.0 ± 4.4	37.3 ± 6.4	71.2 ± 10.8

Rats received an i.v. dose of 4NP (60 μmol/kg). The rats were placed in metabolism cages and urine was collected on ice for 24 hr. Results are expressed as mean ± SEM; 4 rats were used in each group.

Table 3. The excretion of AAF-N-glucuronide and the glutathione conjugates 1-GS-AAF and 3-GS-AAF in bile and urine of male and female rats

	Body weight (g)	Total radio activity	AAF-N glucuronide	1-GS-AAF	3-GS-AAF
		% of the dose excreted during 4 hr			
Males	381 ± 7	65.3 ± 7.4	25.9 ± 2.1	5.4 ± 0.8	7.9 ± 1.1
Females	323 ± 10	67.0 ± 3.1	43.1 ± 2.6†	1.3 ± 0.5*	1.8 ± 0.7*

The rats received an i.v. dose of [ring-^{3}H]-N-OH-AAF. Bile and urine was collected on ice during 4 hr. The amounts of AAF-N-glucuronide, 1-GS-AAF and 3-GS-AAF in each fraction were determined by HPLC with on-line radioactivity detection. The total excretion of AAF-conjugates was calculated from the amounts excreted in the separate fractions. Results are expressed as mean ± SEM; 4 rats were used in each group. * Means significantly different from males at $P < 0.015$ by Fisher's randomization test [15]; † the same at $P < 0.005$.

N-OH-AAF itself because this metabolite is very unstable and immediately reacts with nucleophilic groups in its environment once it is formed [11]. However, the amount of N-OH-AAF available *in vivo* for (amongst others) sulfation can be determined indirectly by measuring the formation of the N,O-glucuronide conjugate [7], the product of the "competing" pathway. Also, a difference in the amount of AAF-N-sulfate formation in males or in females *in vivo* is indicated by the excretion of 1-GS-AAF and 3-GS-AAF because these glutathione conjugates are formed by the non-enzymatic reaction of AAF-N-sulfate with glutathione [12].

Less AAF-N,O-glucuronide was excreted by adult male rats than by females; however, the males excreted more of the AAF-glutathione conjugates than females. Thus, male rats most likely metabolize N-OH-AAF to a much greater extent by sulfation than females (4 times as much; based upon the excretion of glutathione conjugates). This is in agreement with the results of DeBaun *et al.* [3, 13] who found no sulfotransferase activity towards N-OH-AAF in female rat livers *in vitro*. The differences in sulfotransferase activities were reflected in the amount of radiolabelled N-OH-AAF that became covalently bound to hepatic macromolecules *in vivo*, indicating that sulfation played an important role here. Similarly, a much higher covalent binding of labelled N-OH-AAF to rat liver macromolecules in the male rat liver than in the female rat liver is reported in this study.

Thus, N-OH-AAF is sulfated to a high extent only by male rats while 4NP and phenol are equally well sulfated by males and females. An explanation may be that a sulfotransferase iso-enzyme that catalyzes the sulfation of N-OH-AAF is deficient in female rat

liver, while, at the same time, the contribution of this particular iso-enzyme is very insignificant for the sulfation of the phenols. Such an explanation would be consistent with available enzymological data [13, 14].

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